Recent Progress in Bacterial Vaccines: Tuberculosis

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ABSTRACT

Bacille Calmette-Guérin (BCG) is the most widely used vaccine worldwide. However, its efficacy varies from 80% to zero among studies. Meta-analysis of all the published prospective trials and case-control studies indicates approximately 50% efficacy against all forms of tuberculosis, but it is even more effective against the invasive forms of the disease, meningitis and miliary tuberculosis. Geographic latitude accounts for 41% of the variance between studies. The variability between different BCG preparations and the role of environmental nontuberculous mycobacteria are discussed as major factors in the inconsistent results of BCG vaccine trials. New studies to define human genes that code for susceptibility to tuberculosis are reviewed. Despite the great strides being made in identifying vaccine candidates, there is still no reliable surrogate marker of protective immunity to tuberculosis. Human efficacy trials to document prevention of tuberculosis cannot possibly be mounted to test all the vaccine candidates that show promise in animal studies. Recent developments discussed include: the focus on secreted proteins of *Mycobacterium tuberculosis* as vaccine candidates, the genetic differences between BCG and virulent *Mycobacterium bovis*, the ability to create recombinant BCG-expressing cytokines that enhance the immune response and express vaccine candidate antigens, the availability of auxotrophic mutants of BCG as vaccine carriers, and the ongoing debate about other potential vaccine carriers, such as *Salmonella*, vaccinia (particularly modified vaccine Ankara [MVA]) and other avirulent pox viruses that do not replicate in humans.

Key Words: *BCG, tuberculosis, vaccine*

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Tuberculosis (TB) has an estimated world prevalence of 20 million cases. There are 8 million new cases each year, and more than 95% of these are in the developing world. Whereas in developed countries the vast majority of tuberculosis occurs in the age group over 60, in developing countries more than 80% of TB cases occur in the 15 to 59 age group. It is estimated that there are 2 million adult deaths annually from tuberculosis in developing countries, more deaths than are caused by any other infectious agent.[1] Indeed, this tuberculosis mortality accounts for a quarter of all avoidable adult deaths. There has been an increase in incidence of tuberculosis since the start of the human immunodeficiency virus (HIV) epidemic, and in many sub-Saharan countries the number of new cases annually has increased 2 to 5 times in the last 10 years. It is unclear at this stage whether the bulk of these new cases occur as a result of reactivation of dormant organisms or by acquisition of new infection by an immunocompromised host. Undoubtedly both mechanisms occur, and in highly endemic areas where the annual risk of infection is high, it is likely that reinfection accounts for a significant proportion of the new cases.

It is striking that over the past 150 years in the United Kingdom there has been a steady decline in the mortality rate from tuberculosis that cannot be accounted for by scientific advances, introduction of BCG immunization, the advent of chemotherapy, or control programs.[2] There is a significant environmental contribution to the susceptibility of individuals to tuberculosis, including poverty, crowded housing with many people sleeping in one room, and substance abuse, particularly alcohol. Those whose immune competence is reduced are also at high risk, including those taking immunosuppressive agents and steroids, or suffering from malignant disease, HIV infection, diabetes, gastrectomy, or achlorhydria. Pregnancy, lactation, and old age are also risk factors.

PUBLIC HEALTH SOLUTION

Any new effort at introducing a vaccine to replace BCG, has to contend with the convincing argument that public health control of tuberculosis is a remarkably cost effective intervention. This involves passive case detection, sputum microscopy, and short-course chemotherapy, which costs less than $10 per discounted year of healthy life gained.[3] Moreover, in countries where an effective tuberculosis control program has been instituted (Tanzania, Malawi, Benin, and Vietnam) this package has proved remarkably effective, with more than 80% cured and less than 3% failures.[4] It is not surprising that those who have to be responsible for national budgets consider this an effective package that requires their financial backing, a focused effort, and improved management strategies. It would be short-sighted, however, to imagine that *Mycobacterium tuberculosis* will be eliminated using these public health control program strategies alone.

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Before the advent of chemotherapy, the case fatality rate of untreated tuberculosis was about 50%. In 1939, the case fatality of smear positive tuberculosis was 44% at 1 year, 61% at 3 years, and 66% at 5 years. It is clear, therefore, that not everyone dies from this disease and that a third or more of those who develop tuberculosis self-heal. Although traditional wisdom teaches that those who self-heal develop a solid immunity to reinfection, it is difficult to find clear evidence for this. Recurrent disease has been assumed to be due to reactivation, rather than reinfection. The advent of mycobacterial DNA finger-printing now allows this question to be answered. In developing countries, the standard public health assumptions are that an untreated smear-positive case of tuberculosis infects 20 contacts over 2 years. Only two of these 20 develop active disease, one of whom will be smear-positive.[5] Public health efforts focus on the two who become infected, but it is clear that the other 90% are somehow resistant to tuberculosis. There are many factors that account for this resistance, including genetic factors and acquired immunity.

INCIDENCE OF HIV INFECTION

Those who are immunocompromised by HIV have an increased incidence of tuberculosis, commonly quoted at 10% per year in endemic countries, in contrast to a 10% lifetime risk in HIV negatives. Antonucci et al found that the incidence of tuberculosis in 2695 HIV-infected subjects in Italy was highest in those who had low CD_4 counts or evidence of advanced HIV disease, or a past history of tuberculosis, and those who had a positive skin test or who were found to be anergic on skin testing.[6] Again it is notable that not all those whose CD_4 count drops to very low levels develop tuberculosis, even in developing countries where the exposure to *M. tuberculosis* is close to 100%. Those HIV-infected individuals who develop tuberculosis tend to have a different spectrum of disease from HIV-negative adults. They are less likely to have apical regions of the lung affected, cavitating disease, or positive sputum smears, and more likely to have lower zones of the lung affected, lymph node involvement, and extrapulmonary disease. As the CD_4 count becomes lower, there is less granulomatous response noted histologically. Tuberculous meningitis and miliary tuberculosis occur more frequently in those with lowest CD_4 counts.[7]

GENETIC SUSCEPTIBILITY TO TUBERCULOSIS

In addition to acquired immunity, it is evident that there are resistance genes that contribute both to the outcome of exposure to *M. tuberculosis* and also to the pattern of clinical disease. Early explorers in Africa found no evidence of tuberculosis in the interior of Africa in the 1800s. In the first World War, Senegalese troops died at high rates of tuberculosis. More recently, Stead et al have shown that Blacks have twice the relative risk of newly acquired tuberculosis in nursing homes and prisons in Arkansas.[8] Crowle and Elkins demonstrated that *M. tuberculosis* multiplied faster in the macrophages of Blacks, even more so when cultured in autologous plasma, and this was reversed by adding vitamin D_3 in vitro.[9] Lurie demonstrated genetic resistance to tuberculosis in inbred rabbits that were challenged with inhaled *M. tuberculosis*.[10] One strain of rabbit developed widely dispersed, rapidly progressive disease, whereas another inbred strain encapsulated the inhaled organisms in the lung and did not develop disseminated disease. He noted that BCG protected those with rapid progression more than those with better innate resistance.

Vidal et al have identified a gene that codes for resistance to intracellular parasites, including BCG, in the mouse.[11] This Bcg locus also codes for resistance to leishmaniasis and *Salmonella* infection in mice, and it is abrogated by disruption of the *Nramp1* gene. A human homologue has been identified, but so far there is no evidence that this homologue influences susceptibility to mycobacterial infection.[12]

It is perhaps not surprising that people who carry mutations in these important immune response genes demonstrate variable susceptibility to tuberculosis. Indeed, it is likely that there is multigenic control of resistance to this pathogen. Clearly, development of any new vaccine will have to take into account the diversity of immune response genes. So far no clear pattern has emerged for human leukocyte antigen (HLA)-linked susceptibility to tuberculosis. In south India pulmonary tuberculosis was associated with HLA-DR2.[13] It seems unlikely that peptide vaccines would be an effective strategy against tuberculosis that is likely to require a multicomponent vaccine to augment a multipronged attack against this intracellular organism.

BACILLE CALMETTE-GUÉRIN

In 1906 oral infection of guinea-pigs with weakly virulent equine tubercle bacillus led to persistent infection in lymph nodes and conferred resistance to reinfection by the intravenous route. A virulent bovine strain had been isolated by Nocard from a heifer with tuberculosis mastitis, and after 39 passages, a change in the colony morphology in culture was noted.[14] Over the next 13 years, this avirulent mutant was tested in many species and offered protection against tuberculosis without reversion to virulence. Vaccine (BCG) was given to 969 children of tuberculous mothers orally on days 3, 5, and 7; only 3.9% of them died of tuberculosis compared with 32.6% of the unimmunized children. The vaccine was introduced in 1928.[14]

A disaster occurred in 1929 in Lubeck, Germany, where 251 children were given BCG prepared locally and 72 of them died. Investigation revealed that the children had inadvertently been given a virulent strain of *M. tuberculosis*, and although BCG was vindicated, this disaster held up widespread implementation of BCG immunization. It is remarkable to consider that not all the children died from this disastrous mistake, and 179 did not. It would be particularly interesting to reexamine the resistance factors in those who survived.

Globally BCG has been given to more people than any other vaccine, with an estimated 100 million newborns receiving BCG annually; a total of 3 billion doses distributed worldwide is widely quoted.[1] There are several advantages of BCG: it can be given at birth as a single inoculum, which produces long-lasting sensitization. It is safe, stable, produces a scar, and is inexpensive. Initially BCG was given orally, but the organism is killed by acid and tended to cause cervical adenopathy when given by this route. The World Health Organization recommend intradermal administration, since subcutaneous injections give a higher incidence of abscesses and lymphadenopathy. Aerosol administration has been shown to protect guinea-pigs and has also been given safely to human volunteers. There is some evidence to suggest that a low dose might be preferable to high doses of BCG, because the low doses stimulate a protective T helper 1 (Th1) type immune response.

At least 10 control trials of BCG have been conducted worldwide, demonstrating remarkably variable protective efficacy,[15] from 77% in British schoolchildren[16] to no effect in the southern United States and in the large Chingleput study in south India. The vaccine conferred clear benefit against disseminated childhood tuberculosis and against tuberculous meningitis (64% protection). Regression analysis of BCG efficacy has examined the possible effect of various contributing factors. Geographic latitude accounted for 41% of the between-study variance, according to random effects regression analysis of prospective studies.[17] Possible contributing factors for this effect of latitude were considered, including socioeconomic factors, genetic variables, climate, sunlight, diet, virulence of *M. tuberculosis* strains, BCG storage and viability, and the prevalence of environmental mycobacteria. Of these various factors, two are discussed.

Vaccine Variability

The BCG vaccine that is used in different parts of the world is not standardized. Indeed it is produced in several different laboratories and has been shown to have marked genetic variability, and at times even the cultural characteristics, growth rate, morphology, antigen expression, and viability have changed. In 1966, three parent strains accounted for 90% of the vaccine worldwide (Glaxo 1077, Tokyo 172, and Pasteur 1173 P2). To reduce the variability, it was suggested that seed lots should be frozen and subjected to only 12 passages. The organism is grown in liquid culture, which is then homogenized, freeze dried, and resuspended. There is a wide variation in the number of viable organisms per mass of bacteria, such that 10^8 bacilli per milligram BCG yield only 5 to 45 x 10^6 colony-forming units. The variability of vaccines that are given to children is amplified by the problems of storage of BCG. Tropical sunlight for 5 minutes reduced viability of BCG by 99%. In contrast, Danish sunlight for 5 minutes reduces viability of BCG by less than 50%. Storage at 37°C for 1 week reduced viability of BCG by about 75% as compared with storage at 4°C.

UNICEF undertakes the purchase of large lots of vaccine from different manufacturers and then distributes it worldwide. Often those who administer the vaccine are unaware of its origins or of the variable strength of different types of BCG. During the late 1980s in various Central African countries, the supplier of BCG changed from Glaxo to Pasteur, leading to an outbreak of axillary and cervical lymphadenopathy and abscesses. At the time, these events were investigated as a potential complication of HIV infection, but no such association was found. When the inoculum size of the Pasteur vaccine was reduced, the problems disappeared.

Environmental Mycobacteria

A second major variable that is always brought up as a reason for differences of efficacy of BCG by geographic latitude is the prevalence of environmental, nontuberculous mycobacteria. The evidence for this is strong. In the 1940s, skin tests in 14,000 nurses throughout the United States showed large variance in positivity rate, between 14% and 70%, the highest frequency being in the south-eastern states. Tuberculin skin tests (PPD/B) of army recruits for *Mycobacterium avium* yielded a 33% positivity that varied from 20% in the northern states to 80% in the southern states. Further evidence comes from experimental studies in guinea pigs and mice immunized with *Mycobacterium kansasii*, which were 80% as protected from *M. tubeculosis* challenge as with BCG immunization.[17] The most compelling data came from one million American navy recruits who were skin tested with both PPD derived from *Mycobacterium bovis* (PPD/S) and that from *M. avium* (PPD/B).[18] The incidence of tuberculosis between 1958 and 1969 was 49 per 100,000 for the total population. Those who had a strongly positive Mantoux reaction to PPD/S had an incidence of 382 per 100,000. This incidence was slightly lower (273 per 100,000) if they had a smaller PPD/S response (6–11 mm). However, if they had a larger response to the *M. avium* (PPD/B) skin test antigen than to PPD/S the incidence was cut to 29 per 100,000, similar to the level found in PPD/S negative subjects. These

data suggest a strongly protective effect of environmental mycobacteria against the development of tuberculosis.

More recently Stanford and Grange have used an isolate of *Mycobacterium vaccae* derived from a soil sample in Uganda as a nontuberculous mycobacterial vaccine.[19] They have evidence that killed *M. vaccae* induces a delayed hypersensitivity reaction without the more aggressive necrotic response that can sometimes be obtained with BCG. Indeed they have now launched a company to make *M. vaccae* under good microbiologic practice for use in humans, and a large trial is under way in South Africa to test its efficacy at reducing the duration of chemotherapy in patients with tuberculosis.

TUBERCULIN SKIN TEST CONVERSION

Although BCG is used widely throughout the world, it is not used routinely in the United States or in Holland, where the incidence of tuberculosis is sufficiently low for BCG immunization not to be cost effective. Moreover, the administration of BCG complicates the interpretation of subsequent Mantoux tests. Thus, in the United States, contacts of tuberculosis patients whose Mantoux skin tests convert from negative to positive are assumed to be infected and are given prophylaxis with isoniazid for a year. In contrast, this practice cannot be applied in tuberculosis-endemic countries, where Mantoux skin tests are positive in up to 80% of the population by the age of 15.

Analysis of the results of the Medical Research Council (MRC) trial in Great Britain led Hart and Sutherland to conclude that "with highly effective tuberculosis vaccines, the degree of protection conferred on the individual is independent of the degree of tuberculin skin test sensitivity induced in that individual by vaccination." Similarly, a trial of BCG in the southern regions of India demonstrated little protection from BCG despite good vaccine-induced tuberculin conversion. However, it is still widely assumed that, because the skin tests of some individuals convert to positive after BCG immunization, this somehow reflects a surrogate marker of protective immunity. It is also clear that those who have a strongly positive skin test are the ones most likely to develop tuberculosis. Thus, the category of individuals who may be innately most resistant to infection could be those who maintain a negative skin test despite exposure. In every group of health care workers who spend a lot of time working with patients with tuberculosis there are a few who remain skin-test negative despite this occupational exposure.

The whole debate about skin-test conversion and risk of tuberculosis has come to a particularly heated debate in the United States during the past few years when it is claimed that skin tests of the medical house staff looking after patients in New York were converting from negative to positive at the rate of 10% per year. Moreover a decision analysis comparing tuberculin screening strategy with BCG vaccination concluded that BCG unequivocally leads to fewer cases of tuberculosis over 10 years. Moreover, BCG requires only an efficacy rate of 13% to prevent more cases than current strategy. The conclusion was that BCG vaccine should be considered for tuberculin negative house officers and medical students working in high risk areas in the United States.[20] Although this conclusion is hotly debated by other experts, opinions remain divided about the value of immunization with BCG in a country with low prevalence of disease, such as the United States.

WORK TOWARD A NEW VACCINE TO REPLACE BCG

The variability of different batches of BCG and the differences in protective efficacy associated with the rising incidence of tuberculosis throughout the world demand a review of current vaccine strategies against tuberculosis. Within the next couple of years the sequence of the entire *M. tuberculosis* genome will be complete. The genomic sequence of every protein produced by the organism will be known. It will be possible to screen, in recombinant live organisms, a large number of potential vaccine candidates for protection in animal studies. Unfortunately available animal models do not mirror the situation in humans. However, they provide a much more realistic method of screening vaccine candidates. This task is now under way, mainly using the mouse model. However, guinea pigs and rabbits are reasonable alternatives, and the pig is more similar to humans in susceptibility and immune responses.[21]

The major problem in humans is to define protective immunity. Surprisingly this is not yet understood. Surrogate markers of effective immunity are not available, although this is a high-priority area for research. Large population studies are expensive and take a long time to generate data on protective efficacy. It would be impossible to test every promising vaccine candidate in prospective randomized blinded efficacy studies.[22] The definition of surrogate markers of protective immunity is now a high priority.

PROTECTIVE IMMUNITY

The dogma that a positive skin test to tuberculin reflects protective immunity requires further analysis. Those who have a negative skin test response are more susceptible to tuberculosis than those who have an intermediate response. However, there is a U-shaped curve of susceptibility,[23] with highest susceptibility found in those with large Mantoux responses of 15 mm or more.

Current dogma suggests that an effective immune response against *M. tuberculosis* depends on the stimulation of the Th1 arm of the immune response generating interferon γ, which stimulates killing of intracellular mycobacteria. Anything that deviates the immune response toward a Th2 direction would be detrimental.[24] There is evidence that those with HIV infection tend to exhibit a Th2 response, as do those with allergies. However, there are no data to suggest that atophic individuals are more susceptible to tuberculosis, so this hypothesis cannot be entirely sustained. The Th1 response induces effective granuloma formation, to contain the bacteria, but this granulomatous response also depends on tumor necrosis factor, which causes fever and weight loss if produced in excess.

Cytotoxic T cells may play a critical part in effective immunity against tuberculosis. In experimental studies knock out animals that have lost genes important for class I and class II responses have succumbed to tuberculosis, suggesting that both these pathways are important for protection. The generation of cytotoxic lymphocyte responses requires activation of lymphocytes bearing class I major histocompatibility complex molecules of the CD_8 phenotype. To activate these cells, the antigen is processed via the endoplasmic reticulum, whereas class II activation is generated via degradation of protein in the phagolysosome. Whereas BCG tends to remain within the phagolysosome and therefore generates mainly class II responses, *M. tuberculosis* is more virulent and escapes out of the phagolysosome and into the cytoplasm, thereby generating class I responses also. However, this is not the whole story, because experimentally, class I responses have been found in BCG immunized individuals, suggesting that secreted proteins might escape out of the phagolysosome and into the cytoplasm, where they stimulate a class I CD_8 T-cell response. It is assumed that such cytotoxic T cells kill infected macrophages and release the live mycobacteria, which are then phagocytosed and killed by freshly recruited macrophages from other sites.

It has long been assumed that antibodies are totally ineffective in tuberculous immunity despite being produced in large quantities in infected individuals. These high antibody concentrations are generated through a Th2 response and by polyclonal T-cell independent immune responses. Whether antibodies interfere with effective killing of organisms or opsonize organisms for phagocytosis remains unclear, although there is evidence that mycobacteria are internalized through complement receptors.

Antigens of *M. tuberculosis*

The most immunogenic antigens in terms of antibody responses have been the stress or heat shock proteins, produced as part of the stress response induced by phagocytosis and internalization. Much attention has been paid to these proteins over the past 10 years, and particular interest has centered on the stimulation of autoantibodies in response to some of these mycobacterial heat shock proteins. Recently, particular interest has centered around the 6 kD and 30–32 kD secreted proteins of *M. tuberculosis* and how these differ between BCG and virulent *M. tuberculosis*.[25]

Most pathogens have multiple genes responsible for virulence, highlighting the redundancy of different methods by which the organism can enter and survive in a hostile environment within the host. For instance, the *Inv* A gene from *Yersinia pestis* has been found to have some (17%) homology with a mycobacterial invasin. In *Salmonella* there are at least 30 different proteins recognized when cultured in macrophages rather than in media alone.

Mahairas et al have defined the genetic differences between *M. bovis* BCG and virulent *M. bovis*.[26] Using subtractive genomic hybridization to identify genetic differences, they noted three distinct genomic regions. One of these, RD1, is a 9.5 kb DNA segment found to be deleted from all BCG substrains but conserved in all virulent isolates of *M. bovis* and *M. tuberculosis* tested. The reintroduction of RD1 into BCG permitted the expression of at least 10 proteins and resulted in a protein expression profile almost identical to that of virulent *M. bovis*. They conclude that RD1 is involved with regulation of multiple genetic loci and that the loss of virulence of BCG is attributable to a regulatory mutation. This particularly interesting definition of the attenuation of BCG offers the opportunity to use live BCG for a vaccine carrier. Indeed, considerable effort has been made to introduce into BCG genes for better protection against tuberculosis and for augmenting the immune response. Murray et al have shown that recombinant BCG strains that secrete cytokines are more effective at stimulating immune responses against BCG in mice.[27] This may be a particularly promising way of enhancing immune responses to mycobacterial antigens. Others have generated auxotrophic variants of BCG.[28] These live attenuated BCG organisms do not survive in immunocompromised animals, but protect against virulent *M. tuberculosis*. This may be particularly important when considering the immunization of immunodeficient individuals, such as the large HIV-infected population at risk of tuberculosis. Because live attenuated organisms do not multiply in the human host, a safer vaccine is envisaged that can be used in HIV-positive individuals.

Other approaches to vaccine development include the production of recombinant attenuated pox viruses or *Salmonella* vectors into which have been cloned immunodominant antigens from mycobacteria that stimulate CD_4 and CD_8 T-cell responses in those immunized. Some of the attenuated pox viruses do not multiply within the human host and therefore resemble the

auxotrophic mutants of BCG. They have the other advantage of being well-tested in humans over 200 years since the introduction of cowpox vaccination by Jenner. The Ankara strain of modified vaccinia seems particularly promising (Blanchart T, Smith G, et al. Personal communication). This approved vaccine stain of vaccinia has been safely given to many people during the smallpox eradication campaign. Recently it has been shown to lack some of the cytokine receptors that confer resistance to host immune responses.

As mentioned previously, atypical mycobacteria are protective against tuberculosis, and *M. vaccae* is currently of special interest. Other atypical mycobacteria also have shown protective efficacy against tuberculosis, including *M. microti,* in the British BCG trial,[16] and *Mycobacterium W,* in India.

Horowitz et al have protected animals with a combination of secreted proteins in a subunit vaccine.[29] This approach has the advantage that the vaccine is not a live vaccine and is less susceptible to environmental variables, storage, and growth than a live vaccine. However, it suffers the disadvantage of genetic variation in immunologic responses between different individuals. Once the important antigens for protective immunity have been defined, it may be possible to introduced the genes for these antigens into vaccine carriers that are able to deliver multiple vaccines within one organism. These so-called multivaccines offer a particularly attractive prospect. The one-time vaccine given at birth or shortly afterward would be an enormous advantage from a practical point of view of delivery of vaccine in developing countries.

Perhaps the most exciting development has been the use of naked DNA vaccines,[30] in which DNA is injected into muscle and induces the synthesis of relevant vaccine antigens by the host cell. The expressed proteins induce a protective immune response, including antibodies and cellular immune and cytotoxic responses. However, there are regulatory concerns about naked DNA vaccines that have already been shown to be effective in protecting animals against tuberculosis.[31] There is the unsubstantiated danger that DNA will be randomly inserted into the host genome and induce cancers or other problems at the site of inoculation. It is unclear how these concerns will be allayed.

TESTING VACCINES

Perhaps the most challenging future scenario to consider is how to test human vaccines, of which there appear to be a proliferating number of candidates.[32] Surrogate markers of protective immunity are required as well as susceptible populations with a high incidence of tuberculosis in which the most promising candidates can be tested in prospective double-blind randomized trials of efficacy.[33] These trials are expensive and require huge investments in human resources as well as expertise and time to reach conclusions. Consideration of all vaccine candidates for phase 3 trials is not affordable, and urgent attention needs to be given to the way in which the most promising candidates are selected for efficacy trials.

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